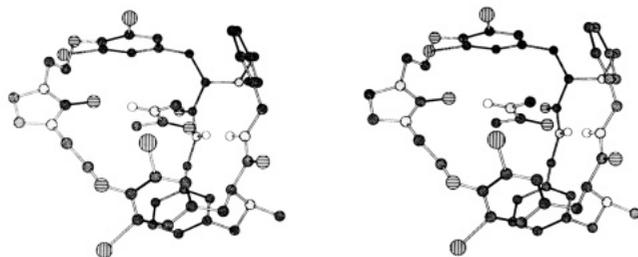


Table I. Free Energies of Association for **1b** and Amides in C₆D₆

| substrate | binding energy, kcal/mol (enantiomer) | saturation achieved, % | enantioselection: $\Delta\Delta G$, kcal/mol |
|-----------------------|--|----------------------------------|---|
| MeNHCOMe | -3.17 | 65 | |
| MeNHCOBn | -2.18 | 62 | |
| BnNHCOH | -3.24 | 64 | |
| BnNHCOMe | -2.84 | 48 | |
| BnNHCOCF ₃ | no complex observed | | |
| BnNHCOEt | -2.33 | 67 | |
| PhCHMeNHCOMe | -3.04 (<i>S</i>), -2.62 (<i>R</i>) | 56 (<i>S</i>), 67 (<i>R</i>) | 0.42 |
| PhCHMeNHCOH | -3.18 (<i>S</i>), -2.85 (<i>R</i>) | 57 (<i>S</i>), 48 (<i>R</i>) | 0.33 |
| PhCHMeNHCOEt | -1.80 (<i>S</i>), -1.55 (<i>R</i>) | 56 (<i>S</i>), 45 (<i>R</i>) | 0.25 |
| 1-NpCHMeNHCOMe | -2.56 (<i>S</i>), -2.31 (<i>R</i>) | 57 (<i>S</i>), 51 (<i>R</i>) | 0.25 |
| BnOAlaNHCOMe | -2.29 (<i>S</i>), -1.81 (<i>R</i>) | 64 (<i>S</i>), 50 (<i>R</i>) | 0.48 |
| MeOPGlyNHCOMe | -1.91 (<i>S</i>), -2.06 (<i>R</i>) | 44 (<i>S</i>), 45 (<i>R</i>) | -0.15 |

namics⁵ starting from the two conformations of **1** observed by X-ray crystallography. In these simulations, the benzyl groups of **1b** were replaced by methyls. After energy minimizing using the OPLS/AMBER force field⁶ with *N*-methylacetamide in the binding cavity, we carried out 250 ps of molecular dynamics at 300 K. The average potential energy stabilized within the first 50 ps. Simulated annealing to ~50 K over 100 ps and energy minimizing gave the final conformers. The conformer of the complex derived from the **1b** crystal structure was found to be more stable by 2.5 kcal/mol in steric energy. When the rigid rotor/harmonic oscillator approximation is used, it is also higher in entropy by 8.8 cal deg⁻¹ mol⁻¹ than the **1a**-derived complex and thus is 5.1 kcal/mol more stable in free energy at 300 K. Its stereostructure is shown below:



As revealed in the structure above, the atoms bearing hydrogens that display the described NOE signals are indeed close in space. Furthermore, the observed coupling constants for hydrogens of the diiodotyrosine α and β carbons in the complex ($J_{\alpha,\beta} = 2.8$ and 9.2 Hz) are similar to those calculated by using Altona's equation⁷ (1.4 and 9.8 Hz). If the **1**/amide complex has the geometry shown, then we would expect selective binding with the amides of primary amines having nitrogen attached to a chiral center of the *S* configuration.⁸

As summarized in the table, we do indeed find enantioselective binding of **1b** with certain chiral amides. Binding energies were measured by NMR titration, and error propagation analysis gives error limits of ± 0.1 kcal/mol. While the chiral binding differences are not large, they lie well outside the error range of the measurements. Except for the acetamide of phenylglycine (PGly) methyl ester, which has substituents having similar steric demands,⁹ it is the *S* enantiomer that binds more tightly. Distinctions between amide enantiomers were also observed by ¹H NMR. With PhCHMeNHCHO, for example, signals from the two enantiomers for the chiral methine hydrogen and the formamide C–H and N–H separated by >0.1 ppm upon treatment with **1b**.

It should be easy to design chiral hosts that bind enantiomeric guests with significantly different association energies because the thermodynamics of enantiomeric complexation are relatively

simple. Enantiomeric guests have identical solvation energies, and differences in binding energies result exclusively from the relative stabilities of the complexes. In contrast, differences in the solvation energies of nonenantiomeric guests can have a major effect on selectivity.¹⁰ Nevertheless, many previous reports of chiral hosts note little detectable difference in the energies of diastereomeric complexes. A likely explanation is that many different conformations of complexes are involved. In our host, cyclophane linkages, bridged macrocyclic structures, and C₂ symmetry all operate to reduce but not eliminate conformational heterogeneity. Further rigidification is clearly desirable and should provide enhanced enantioselection.¹¹

Supplementary Material Available: Stereopair plots of the X-ray structures of **1a** and **1b** (1 page). Ordering information is given on any current masthead page.

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(11) This work was supported by NSF Grants CHE86-05891 and CHE89-11008.

Proline Assignments and Identification of the Cis K116/P117 Peptide Bond in Liganded Staphylococcal Nuclease Using Isotope Edited 2D NMR Spectroscopy

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Proline is usually the most difficult type of amino acid residue to assign in a protein because the pyrrolidine ring lacks an amide proton, and therefore the essential sequential connectivities involving this proton are absent.^{1,2} Although connectivities involving the proline δ -protons can substitute for the lacking amide proton connectivities,^{1,3} the δ -protons are often difficult to identify because

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*To whom correspondence should be addressed.

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Therefore the connectivities involving the Gly55 α -protons and the P56 γ -protons are obscured by the intense β -proton connectivities in Figure 1b,c.

The long-range connectivities in Figure 1, that link Pro residues to remote residues in the sequence, are consistent with reported⁴ NMR assignments and the distances calculated from the crystal structure.⁷ These results are further evidence that there is a close correspondence between the solution and crystal structure of the ternary complex throughout most of the protein, as concluded previously.^{4,10,11}

The isotope editing approach described herein should be a generally useful method of assigning proline spin systems in moderate size proteins. Labeling with [3,5-¹³C₂]proline should improve the efficiency of the method, because all relevant information could be obtained by using one double-labeled sample. The double-labeled proline could be synthesized by following the scheme used to obtain [4-¹³C]proline.¹²

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Registry No. Nase, 9013-53-0; Pro, 147-85-3.

Supplementary Material Available: Spectra (500 MHz ¹H/¹³C HMQC) of Nase labeled with [2-¹³C]Pro and [4-¹³C]Pro and spectrum (500 MHz ¹³C edited NOESY) of Nase labeled with [4-¹³C]Pro (3 pages). Ordering information is given on any current masthead page.

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Observation and Sequence Assignment of a Cis Prolyl Peptide Bond in Unliganded Staphylococcal Nuclease

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Cis-trans isomerization of X-prolyl peptide bonds is now considered as both a major determinant of the rate of protein folding and an explanation for the existence of multiple folded forms of proteins in solution.¹⁻³ Indirect evidence for these effects of prolyl peptide bonds has been recently obtained by examining the properties of mutant proteins in which specific prolyl residues have been replaced by site-directed mutagenesis techniques. For

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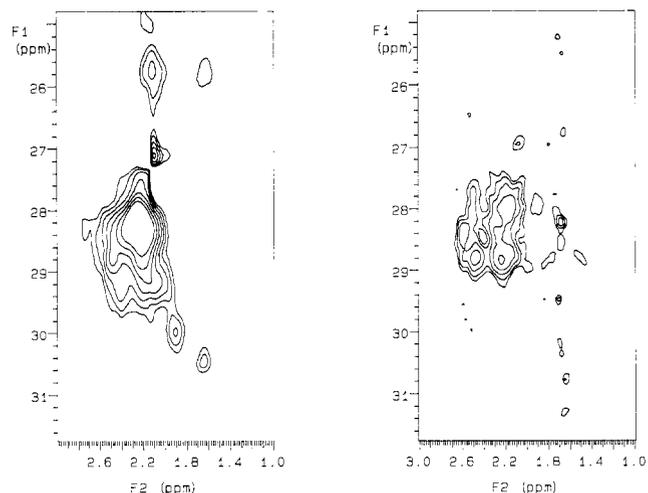


Figure 1. ¹H-¹³C heteronuclear chemical shift correlation spectra of wild-type SNase (left panel) and its P117G mutant (right panel) labeled with [4-¹³C]proline. The data were obtained in proton detection experiments of ¹H-¹³C chemical shift correlations at pH 5.5 and 40 °C.¹⁷ Assignments are made in the text.

example, by investigating substitutions for specific proline residues, Richards and co-workers have deduced that isomerization of a single prolyl peptide bond is the rate-determining step in the folding of thioredoxin from *Escherichia coli*.⁴ Also, Dobson and Fox have implicated cis and trans isomers of a single prolyl peptide bond in staphylococcal nuclease (SNase) as the explanation for the coexistence of multiple folded forms of the protein.⁵⁻⁷ However, the conclusions reached in these and all other solution studies of isomerization of prolyl peptide bonds have not been based on the observation of cis prolyl peptide bonds but on the effects of this putative isomerization on biophysical properties such as the rates of folding and the ¹H NMR spectra of the resolved histidine H_i resonances. In this communication we report observation and assignment of selected ¹H and ¹³C NMR resonances of a single proline cis X-prolyl peptide bond in SNase.

A number of studies of the resonances of the resolved H_i protons of the four histidine residues in SNase have suggested the presence of an equilibrium mixture of two monomeric folded forms since two resonances are detected for each of these protons.⁵⁻¹⁰ Investigation of the temperature dependence of these resonances reveals that the multiplicity is preserved in the unfolded form. In the presence of the active site ligands Ca²⁺ and thymidine 3',5'-bisphosphate (pdTp), the multiplicity of resonances in the folded state is eliminated. Crystallographic studies of SNase complexed with the active-site ligands have revealed that the peptide bond between Lys 116 and Pro 117 is cis whereas the remaining six X-prolyl peptide bonds are trans.¹¹ The ¹H NMR spectrum of the aromatic region of the site-directed mutant in which Pro 117 is replaced with a glycine residue (P117G) reveals only a single resonance for each H_i proton. The simplest explanation for these observations is that the two folded forms of unliganded SNase in solution as detected by ¹H NMR spectroscopy can be associated with a major cis and a minor trans isomer of the Lys₁₁₆-Pro₁₁₇ peptide bond. However, the ¹H NMR spectrum of P117G differs from that of the wild-type enzyme,

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